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Tree diversity reduces co-infestation of Douglas fir by two exotic pests and pathogens

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Abstract

The number of non-native invasive pests and pathogens has increased dramatically in recent years, with disastrous consequences for the health of forests worldwide. Multiple studies have shown that mixed forests may suffer less damage from insect pests than single species forest. This "associational resistance" can be notably explained by the fact that heterospecific neighbours make it more difficult for herbivores to locate and then exploit their host tree. However, the validity of these findings in the case of non-native, invasive pests and pathogens remains to be demonstrated. In this study, we monitored over two hundred Douglas firs in pure and mixed plots of a tree diversity experiment to assess the damage from the non-native gall midge *Contarinia pseudotsugae* and the non-native needle cast *Nothophaeocryptopus gaeumannii*. The probability of Swiss needle-cast infection was lower in Douglas fir trees surrounded by heterospecific neighbours. Gall midge damage was lower on Douglas firs surrounded by taller neighbours, consistent with the hypothesis of reduced host Apparency. Douglas fir trees that were more damaged by *C. pseudotsugae* were also more often infected by *N. gaeumannii*. Our study thus provides partial support of the associational resistance hypothesis of mixed forests against exotic pests and pathogens. Promoting forest species diversity at the stand level could, therefore, offer interesting prospects for reducing the impact of biological invasions, especially those involving both pests and pathogens.

Keywords

Douglas fir, exotic pathogen, exotic pest, resource concentration, tree apparency, tree diversity

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Introduction

In the last decades, climate change and increasing global trade and travel have led to a dramatic increase in invasion by non-native forest insects and pathogens (Parmesan 2006; Jactel et al. 2020). Many of those invasive alien species, such as the emerald ash borer (Poland and McCullough 2006), Asian longhorn beetle (Haack et al. 2010), pinewood nematode (Kim et al. 2020) or Swiss needle cast (Hansen et al. 2000) are often responsible for tremendous damage in forests around the world. Invasive pests and pathogens are of great concern not only because of the severe economic damage they generate (Aukema et al. 2011; Haight et al. 2011; Zenni et al. 2021), but also because they threaten the integrity and vitality of forests, thereby limiting the provision of important ecosystem services, such as climate change mitigation, habitat for forest species, pollination or biomass production (Brockerhoff et al. 2017; Griscom et al. 2017; Mori et al. 2017). Hence, while there is a need to reduce the rate of introduction of non-native species, it is also essential to improve our knowledge of the mechanisms that can help mitigate their damage in forests.

Studies from agricultural systems have shown that plant diversity can lead to a decrease in insect damage (Barbosa et al. 2009). This phenomenon, called "associational resistance", has also received consistent support in forests, where trees are less prone to herbivory when growing in mixtures compared to monocultures (Guyot et al. 2019; Jactel et al. 2021; Ward et al. 2022). Two non-exclusive hypotheses have been proposed to explain associational resistance (Jactel et al. 2021): the natural enemies' hypothesis (Root 1973; Stemmelen et al. 2022) and the resource concentration hypothesis (Hambäck and Englund 2005). The natural enemies' hypothesis states that richer plant communities can favour herbivore's natural enemies by increasing the availability of suitable microhabitats and the amount of complementary prey or alternative resources, such as pollen or nectar and will result in a better control of herbivores. On the other hand, the resource concentration hypothesis states that herbivores will be more likely to immigrate to and less likely to emigrate from monospecific forest patches due to higher resource availability. Another consequence of growing trees in monoculture is that host trees are more accessible, i.e. more easily located by their herbivores. Following the "host apparency" theory (Castagneyrol et al. 2013), host trees surrounded by non-host trees that are taller or emit repellent volatile organic compounds are less likely to be colonised by specialist herbivores (Castagneyrol et al. 2013; Haase et al. 2015).

The mechanisms driving associational resistance effects are further complicated by overlooked horizontal interactions between herbivores and between herbivores and plant pathogens. For example, a meta-analysis by Fernandez-Conradi et al. (2018a) reports that, on average, plant infestation with pathogenic fungi alters host foraging behaviour and reduces insect herbivore performance. Yet, pathogenic fungi are also influenced by tree diversity, which has been well documented in the case of the interaction between oaks and powdery mildew (Field et al. 2020). It follows that any effect of tree diversity on fungal pathogens likely alters the strength and direction of

associational effects on insect herbivores (Field et al. 2020). However, since many plant pathogens are vectored by insects or simply benefit from mechanical injuries made by insect mouthparts to plants, the inverse relationship may also be true. It is unsure whether the same mechanisms that are effective against native herbivores are symmetrically efficient against introduced pests. On the contrary, the fact that introduced species have not co-evolved with trees, herbivores and enemies in their introduced range likely alter the effect of tree diversity (Brockerhoff and Liebhold 2017; Nunez-Mir et al. 2017). For example, biological control can be expected to be enhanced in mixed forests only if native predators or parasitoids are generalist enough to switch to exotic preys (Fernandez-Conradi et al. 2018b). If not, then even an increase in the diversity of herbivores' enemies in mixed stands may not be sufficient to increase biological control of exotic pests. On the other hand, the dilution or diversion effect of host trees by non-host trees in mixed species forests can be expected to be more powerful against exotic herbivores that have not co-evolved with these native tree species.

Contarinia pseudotsugae Condrashoff (Cecidomyiidae) is an exotic invasive forest pest in western Europe, originating from North America where it causes damage on Douglas fir (*Pseudotsuga menziesii* Franco), a coniferous tree species also originating from North America (Condrashoff 1961; Roques et al. 2019). It is part of a complex of three species of needle midge, the others being *Contarinia constricta* Condrashoff and *Contarinia cuniculator* Condrashoff (henceforth often referred to as *Contarinia* spp.). In Europe, it was first reported in Belgium and the Netherlands in 2015, from where it spread to France and Germany in 2016 (EPPO 2019; Wilson et al. 2020). Adults lay eggs within opening buds. Few days later, eggs hatch and larvae enter the needles, inducing the formation of galls. Damaged needles tend to bend, gradually change colour and to fall prematurely. Although it is not considered a mortality-inducing factor on its own, heavy infestation by *C. pseudotsugae* can lead to severe defoliation, in association with other pests or pathogens and could threaten the health of Douglas fir trees or impair their growth (EPPO 2019; Ligot et al. 2020; Wilson et al. 2020).

One of the main pathogens frequently co-occurring with *C. pseudotsugae* on Douglas fir is the fungus *Nothophaeocryptopus gaeumannii* (T. Rohde) Videira, C. Nakash., which causes a foliar disease called Swiss needle cast. Although discovered in Switzerland in 1925, this pathogen is native to North America – hence its misleading common name – and is nowadays present in Europe and Australasia (Hood and Kimberley 2005; Kimberley et al. 2011). Symptoms develop slowly, but lead to chlorosis, necrosis and premature fall of the infected needles (Black et al. 2010; Wilson et al. 2020). It is well known that insects can act as vectors for fungal pathogens, either directly or indirectly by wounding the plants and promoting the infection (Almeida and Purcell 2005). Larvae of *C. pseudotsugae* induce the production of new plant tissue during the cecidogenous process and might alter the ability of fungi to invade needle tissues. However and despite the fact that *C. pseudotsugae* and *N. gaeumannii* symptoms often co-occur on Douglas fir, no study so far has investigated the potential interaction dynamics between the pest and the pathogen.

In this study, we assessed the effect of forest mixtures on damage caused to Douglas fir by the exotic pest *C. pseudotsugae* and the exotic pathogen *N. gaeumannii*, as well as the potential effect of *C. pseudotsugae* damage on the co-occurring infection by *N. gaeumannii*. We used a long-term tree diversity experiment to sample 207 Douglas firs of various height, located in plots of increasing tree diversity from monoculture to four species mixtures. We measured needle damage by the Douglas fir needle midge and infection by the Swiss needle cast to test the following hypotheses: (1) Needle damage by *C. pseudotsugae* and *N. gaeumannii* increases with increasing proportion of Douglas fir in forest plot (host concentration hypothesis); (2) Douglas firs taller than neighbouring trees suffer more needle damage than smaller ones (host Apparency hypothesis); (3) Damage by *C. pseudotsugae* increases the probability of infection by *N. gaeumannii*. In doing so, our study aimed at a better understanding of the ecological factors driving primary and subsequent invasion by exotic pests and pathogens in pure vs. mixed forests.

Materials and methods

Study site and tree selection

The study was conducted in Belgium, six years after the first detection of *C. pseudotsugae* in the country, in the tree diversity experiment FORBIO (Verheyen et al. 2013), belonging to the larger network TreeDivNet (Paquette et al. 2018). Specifically, the experiment took place on the site of Gedinne, located in the Ardennes (Belgium, 49°59'N, 4°58'E) and consisting of two sub-sites ca. 2 km apart (Gribelle and Gouverneurs). At each sub-site, the same pool of five species was planted: sycamore maple (*Acer pseudoplatanus* L.), European beech (*Fagus silvatica* L.), hybrid larch (*Larix x eurolepis*), sessile oak (*Quercus petraea* Liebl.) and Douglas fir. Each sub-site is composed of 42 (Gribelle) to 44 (Gouverneurs) plots of increasing tree species richness, ranging from 1–4 tree species (Fig. 1), with a total of twenty different compositions: all five monocultures, all five possible four-species combinations and a random selection of five two- and five three-species combinations (For more details on plot composition, see Verheyen et al. (2013)).

Tree species were planted at an equivalent proportion in mixtures (e.g. 50:50 in two-species mixtures). Even in mixture, trees were planted in monospecific patches of 3 × 3 trees, with patches arranged in a checkerboard pattern in the two-species mixtures and randomly distributed in the three- and four-species mixtures. Therefore, individual trees were surrounded by a varying number of conspecific neighbours, even in mixed plots in which the central tree of each monospecific patch was always surrounded by conspecific neighbours. Finally, four subplots were delimited in each plot and consisted in a 4 × 4 tree patches, where tree height and circumference are measured yearly.

In each of the 20 plots containing Douglas fir (10 plots in each sub-site), we randomly selected three of the four sub-plots in which tree height and circumference had been measured in the current year (2021). We sampled every Douglas fir present

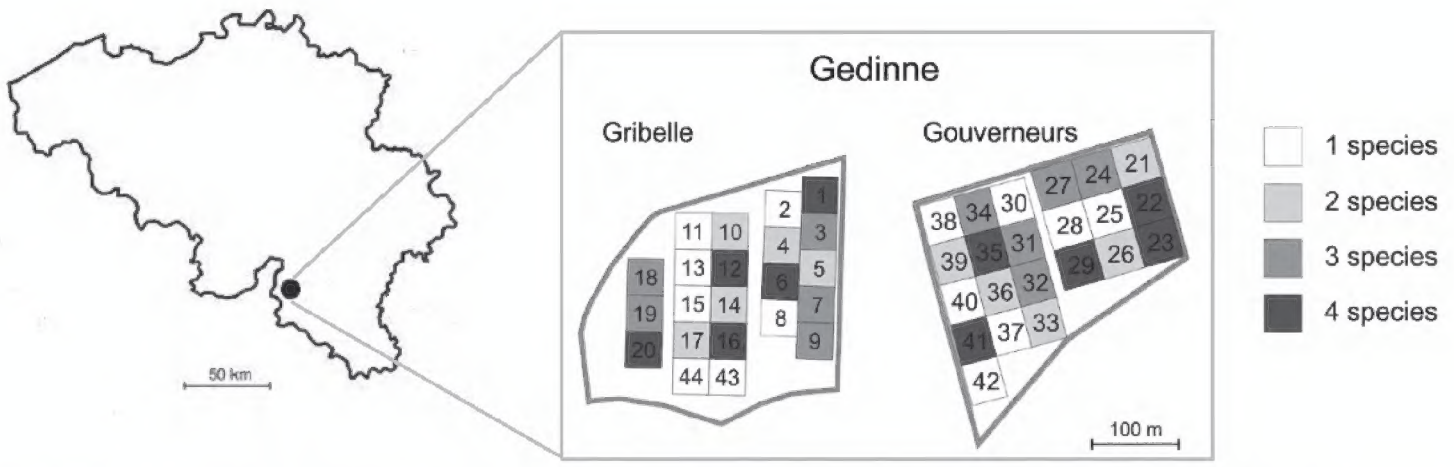

Figure 1. Map of Belgium with the location and experimental set-up of the FORBIO Gedinne sub-sites. The tree species diversity per plot ranged from one species (white) to four species (dark grey).

in those subplots, for a total of 207 trees. Finally, we calculated the mean height difference between each selected Douglas fir and their direct neighbours, regardless of whether they were other Douglas firs or trees of the other three species (henceforth called "Apparency"), setting tree height to zero when neighbours of sampled Douglas fir were missing (dead).

Needle damage by *Contarinia pseudotsugae*

On each sampled Douglas fir, we selected a branch, at mid-height of the tree crown and collected five current-year shoots, starting with the terminal shoot, then picking up every second lateral shoot down to the base of the branch. Shoots were then placed in paper bags, sealed and sent to Bordeaux (France) for damage assessment by a single observer (AS), blind to treatment (plot) identity.

We estimated "needle damage" as the percentage of needles on a shoot that turned dark-brown because of the presence of *Contarinia pseudotsugae* galls, following the methodology developed by the Walloon Forest Health Observatory (OWSF). We used an ordinal scale of six percentage classes of damage: 0%; 1 – 10%; 11 – 20%; 21 – 40%; 41 – 60%; 61 – 90%; 91 – 100%, assigning each of the five shoots to one damage class. Finally, we estimated needle damage at the level of individual tree by averaging the median values of defoliation class across the five shoots. Five needles per trees were collected and used to detect the presence or absence of *N. gaeumannii*.

Infection by *Nothophaeocryptopus gaeumannii*

For DNA extraction, the collected needles were placed in 2ml Eppendorf Tubes containing a sterile metal bead (4mm diameter). Tubes were closed with an AirPore tape, stored at -20 °C and lyophilised in an Alpha 2-4 LD plus freeze dryer (Christ, Osterode am Harz, Germany) for 1.5 days. After lyophilisation, the needles were crushed in a MM400 Retsch Mill (Retsch, Haan, Germany) at 30 Hz for 2 min. Further steps in DNA extraction were done using the Sbeadex Plant kit (LGC Genomics GmbH, Berlin, Germany). Lysis was performed by adding 300 µl Lysis Buffer PVP

(LGC Genomics GmbH) containing 40 µl/ml Debris capture beads (LGC Genomics GmbH), 0.3 µg/µl Proteinase K (LGC Genomics GmbH) and 1.3% 1-thioglycol (Sigma-Aldrich, Buchs, Switzerland) to each sample and subsequent mixing until the samples were well suspended. After incubation at 60 °C for 1 hr, samples were centrifuged at 5,700 g for 5 min and 200 µl of lysate added to a 96-well deep-well plate (Thermo Fisher Scientific, Waltham, USA) containing 420 µl Binding Buffer PN (LGC Genomics GmbH) and 10 µl Sbeadex Particles (LGC Genomics GmbH). The following steps were conducted using the automated KingFisherTM Flex Purification 96 System (Thermo Fisher Scientific). After mixing for 30 min, the Sbeadex Particles with the DNA attached were collected and transferred to 400 µl Wash Buffer PN1 (LGC Genomics GmbH) and mixed for 6 min. This step was repeated before the particles were transferred into 400 µl Wash Buffer PN2 (LGC Genomics GmbH). After mixing for 6 min, the particles were transferred to 50 µl Elution Buffer AMP (LGC Genomics GmbH) and mixed for 7 min to elute the DNA attached to the particles. All DNA purification steps were performed at room temperature. The DNA extracts were stored at -20 °C before dilution and further analyses.

To detect *N. gaeumannii*, we performed a multiplex quantitative real-time PCR (qPCR) with a specific primer pair targeting the beta tubulin gene and the corresponding dual-labelled probe (Winton et al. 2002). An universal 18S primer pair with the corresponding dual-labelled probe 18S uni-P (Ioos et al. 2010) was also used, targeting a highly conserved region of the 18S ribosomal rDNA, to assess DNA quality. The concentrations of the universal primers and probes were reduced in the multiplex reaction mixture to avoid any competition for reagents with the other primers and probes. Quantitative PCR conditions established by Winton et al. (2002) were adapted for high-throughput analyses using the qPCR Takyon core kit NO ROX (Eurogentec, Seraing, Belgium). Reaction volumes of 20 µl contained 5 µl of the 1:10 diluted needle DNA extracts, 1× reaction buffer (Eurogentec), 5.5 mM $MgCl_2$ (Eurogentec), 0.4 mM dNTPs (Eurogentec), 0.1 × ROX reference dye (Invitrogen, Waltham, USA), 0.5 µM of the specific forward and reverse primer, 0.15 µM each of the universal primers, 0.3 µM of the specific probe, 0.05 µM of the universal probe, 0.5 U Takyon enzyme (Eurogentec) and LiChrosolv Water (Merck, Darmstadt, Germany). Cycling conditions were 3 min of initial denaturation at 95 °C, followed by 40 cycles of 10 s of denaturation at 95 °C and 60 s of annealing and extension at 61 °C. All qPCRs were performed in a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Rotkreuz, Switzerland). As a standard in the qPCR assay, a plasmid construct (pUC57 derivate, Eurogentec) containing the sequence obtained by the PGBT primers, was used in a 10-fold serial dilution from 5×10^7 to 5 copies per µl. Five µl of the serial dilution were used for each dilution step and standard curve reactions were performed in triplicate.

Explanatory variables

To test the hypothesis that taller trees are more damaged by *C. pseudotsugae* or more exposed to infection by *N. gaeumannii*, we used Douglas fir absolute (Height) and relative (Apparency) height. We also included the density of Douglas fir in the plot and

in the direct neighbourhood – number of Douglas fir trees present amongst the eight nearest neighbors – of a focal Douglas fir tree to investigate the potential validity of the resource concentration hypothesis. Density of Douglas fir corresponded to the number of Douglas fir either in the plot or in the direct neighbourhood of a focal Douglas fir. Finally, as damage by *C. pseudotsugae* could alter the biology of the needle or weaken Douglas fir prior an infection by the Swiss needle cast, we included *C. pseudotsugae* damage as an additional explanatory variable only in the model built to explain the probability of infection by *N. gaeumannii*.

Statistical analyses

We built two different models to test for the effect of explanatory variables on needle damage by *C. pseudotsugae* and infection by *P. gaeumannii*.

First, we used a linear mixed model to test the effect of Douglas fir absolute (Height) and relative (Apparency) height and Douglas fir density in the plot (Density plot) and in the direct neighbourhood (Density neigh.) as well as the interaction between height and Apparency on mean *C. pseudotsugae* damage per tree. Subplots (Subplot_ID), nested within Plot (Plot_ID), were included as random factors to account for spatial autocorrelation. Response variable was square-rooted to satisfy model assumptions of normality and homogeneity of residuals and predictors were scaled. Variance inflation factors were checked for every explanatory variables used in the model and were never > 5, the usual cut-off values used to check for multicollinearity issues (Miles 2014). As the interaction between height and Apparency had no significant effect, we removed it from the final model during the model simplification process.

Second, we used a generalised linear model with a binomial error distribution family to analyse sources of variation in the probability of Douglas fir infection by the Swiss needle cast. We used the same model structure as in Eq. 1, adding *C. pseudotsugae*. damage as an additional covariate. Again, the interaction between height and apparency had no significant effect and we removed it from the final model during the model simplification process. Statistical analyses were performed using R software version 4.1.0 (R Core Team 2022), with the package lme4 (Bates et al. 2015).

Results

The height of the focal Douglas fir studied ranged from 175 to 954 cm, with a mean (± SE) of 559.6 ± 13.0 cm. Mean height (± SE) of European beech, sycamore maple, sessile oak and hybrid larch were 357.8 ± 9.2, 364.0 ± 16.3, 407.6 ± 14.2 and 948.8 ± 13.6 cm, respectively (Supplementary material). Height difference between a Douglas fir and its closest neighbours was on average (± SE) -14.2 ± 13.8 cm, i.e. Douglas firs were on average shorter than their neighbouring trees. Maximum differences were recorded in plots 20 and 22 (Species composition: Beech, maple, larch and Douglas fir, in the Gribelle and Gouverneurs blocks, respectively), where Douglas fir was 400 cm shorter and 520 cm taller than their neighbours, respectively.

Mean needle damage by *C. pseudotsugae* was on average 16.25 ± 1.25% needles attacked, ranging from no damage at all on 12 Douglas firs to 91.5% of damage on the most impacted Douglas fir. Infection by the Swiss needle cast was confirmed on 118 (57%) of the 207 sampled Douglas firs.

Taller Douglas firs (absolute height) suffered significantly more damage by *C. pseudotsugae* than smaller ones (average standardised model coefficient parameter estimate ± standard error: 1.10 ± 0.24, n = 192, Fig. 2, Table 1, model 1). Mean needle damage by *C. pseudotsugae* also significantly increased with Douglas fir Apparency. Douglas firs that were taller than their neighbours suffered from higher damage than Douglas fir that were smaller than their neighbours (0.59 ± 0.22, n = 192, Fig. 3, Table 1, model 1), but the coefficient parameter estimate for this effect was almost twice lower than the one of tree absolute height. Lower Douglas fir Apparency seems to occur more frequently in mixed species plots including *Larix x eurolepis*, the tallest species in the experiment (Supplementary material). None of the other predictors had a significant effect on mean needle damage by *C. pseudotsugae*. Fixed predictors explained 12.1% of the variance in mean needle damage. Fixed plus random predictors explained 19.7% of the variability in mean needle damage by *C. pseudotsugae*.

The probability of presence of *N. gaeumannii* increased significantly with the density of Douglas firs at the neighbouring scale (0.70 ± 0.22, n = 192, Fig. 4B, Table 1, model 2), but not at the plot scale. Additionally, the probability of a Douglas fir being infected by the Swiss needle cast pathogen increased with increasing mean needle damage by *Contarinia* (0.65 ± 0.23, n = 192, Fig. 4A, Table 1, model 2). In this model, fixed predictors and fixed plus random predictors explained 19.6% and 51.8% of the variability in the probability of infection by *N. gaeumannii*, respectively.

Table 1. Summary of model coefficients for the two models presented in this study. Bold characters indicate that parameters are significant. R^2m and R^2c represent R^2 of fixed and fixed plus random factors, respectively.

Models / Parameter	Estimate	Standard error	95% CI	P value	R^2m (R^2c)
Model 1 – Mean needle damage					**0.121 (0.197)**
(Intercept)	**3.47**	**0.19**	**(3.10; 3.83)**	**< 0.001**	
Apparency	**0.59**	**0.22**	**(0.16; 1.04)**	**0.008**	
Height	**1.10**	**0.24**	**(0.63; 1.60)**	**< 0.001**	
Density neigh.	-0.01	0.15	(-0.31; 0.29)	0.919	
Density plot	0.08	0.19	(-0.29 0.45)	0.668	
Model 2 – Infection rate					**0.196 (0.518)**
(Intercept)	0.29	0.36	(-0.48; 1.09)	0.420	
Apparency	-0.12	0.34	(-0.83; 0.53)	0.711	
Height	-0.54	0.42	(-1.46; 0.26)	0.201	
Density neigh.	**0.70**	**0.22**	**(0.27; 1.17)**	**0.002**	
Density plot	0.61	0.37	(-0.15; 1.45)	0.105	
Contarinia damage	**0.65**	**0.23**	**(0.21; 1.16)**	**0.006**	

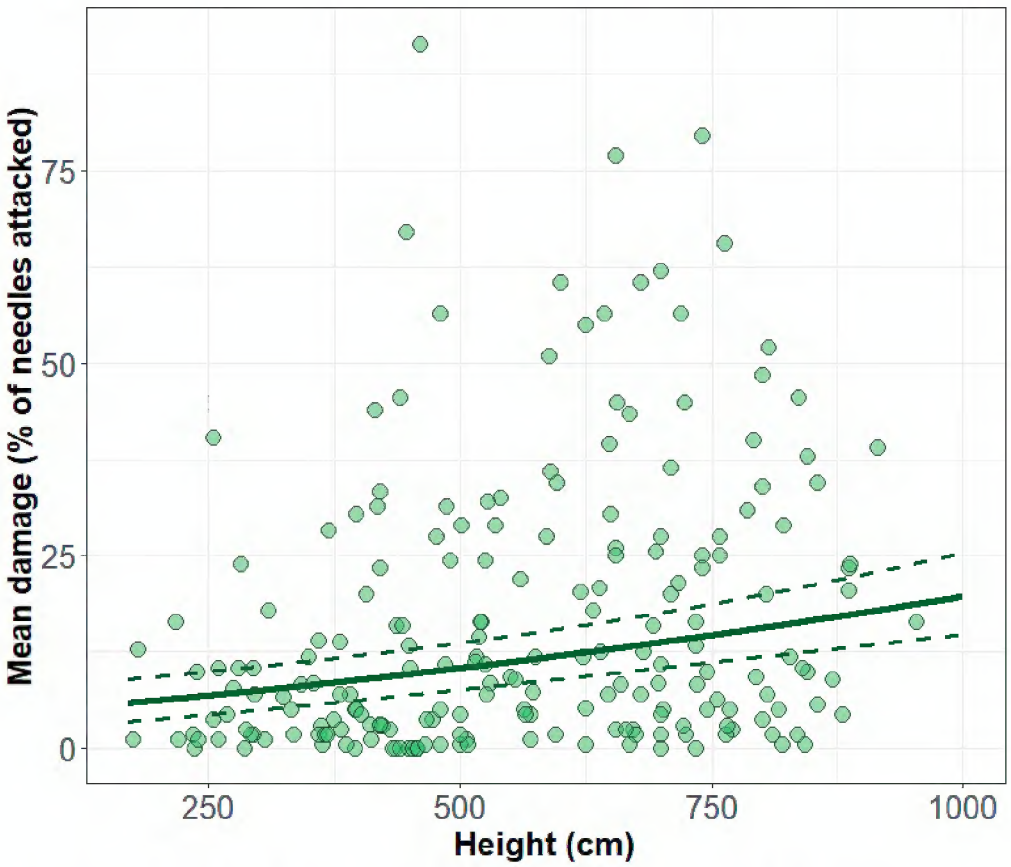

Figure 2. Effect of tree height of a focal Douglas fir on mean damage by *C. pseudotsugae.* Green dots represent individual trees. Solid and dashed lines represent prediction and adjusted standard error of model 1, with values of other model variables set at average (Table 1).

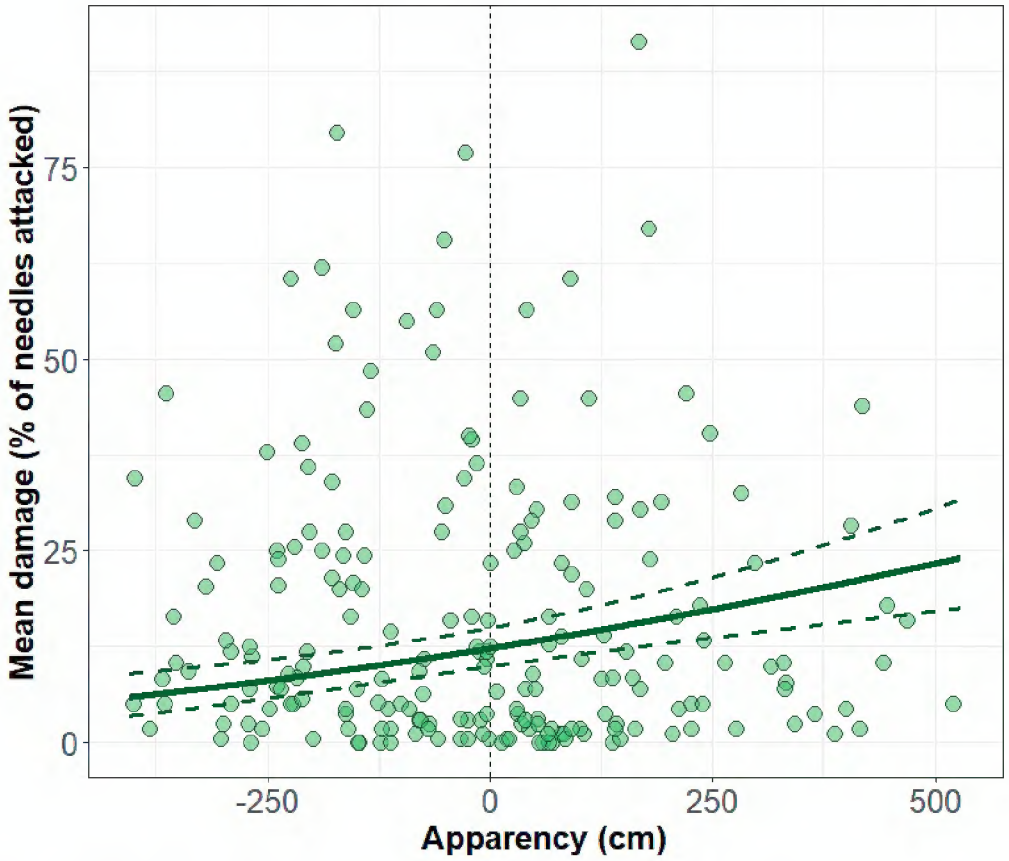

Figure 3. Effect of Douglas fir apparency on the mean damage by *C. pseudotsugae.* Apparency is the mean height difference between a focal Douglas fir and its direct neighbours. Dots at the right and at the left of the vertical dashed line are Douglas fir trees that are taller and smaller than their neighbours, respectively. Solid and dashed lines represent prediction and adjusted standard error of model 1, with values of other model parameters set at average (Table 1).

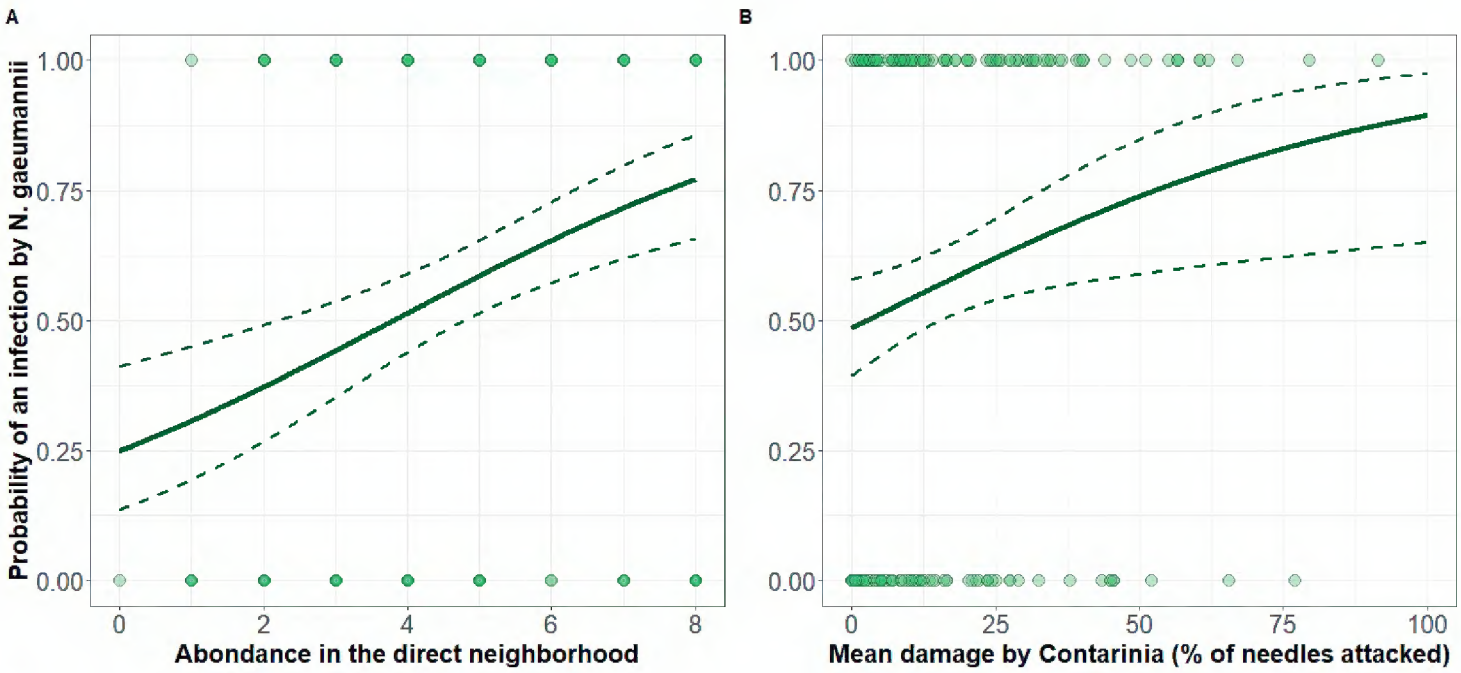

Figure 4. Effects of (**A**) the density of Douglas fir in the direct neighbourhood of a focal Douglas fir and (**B**) of mean damage by *C. pseudotsugae* on the probability of a Douglas fir being infected by *Nothophaeocryptopus gaeumannii*, the causal agent of Swiss needle cast. Solid and dashed lines represent prediction and adjusted standard error of model 2 (Table 1). Dots at probability 0 and 1 represent Douglas fir that were (1) or were not (0) infected.

Discussion

Our study shows that tree height and Apparency were two main drivers of needle midge *C. pseudotsugae* on Douglas fir. Douglas firs taller than their neighbours exhibited more needle damage. We found no effect of Douglas fir concentration, nor at the plot level, nor at the direct neighbours' scale, on damage by *C. pseudotsugae*. However, there was a significant, positive effect of Douglas concentration at the local scale on the probability of infection by the Swiss needle cast fungus *N. gaeumannii*. Douglas fir suffering more needle damage by *C. pseudotsugae* also had higher probability of being infected by the fungal pathogen.

Tree Apparency is a component of tree diversity. The concept was initially coined to describe the probability of a plant being identified by its herbivores (Feeny 1970; Endara and Coley 2011; Strauss et al. 2015). This definition was then refined to include not only the plant's own characteristics (size, colour and odour), but also those of its neighbours, ultimately determining how much a plant is prone to be found by herbivores (Castagneyrol et al. 2013). In this study, we found that more apparent Douglas firs suffered from more needle damage by the Douglas fir needle midge *C. pseudotsugae* than less apparent trees. Taller, non-host neighbouring trees are known to contribute to the disruption of visual (Dulaurent et al. 2012) or olfactory cues (Jactel et al. 2011) used by herbivorous insect. Although no studies so far have been done to identify the mechanisms used by *C. pseudotsugae* to locate Douglas fir trees, it has been shown that midge of the same *Cecidomyiidae* family are using both visual and olfactory cues to find potential hosts (Harris and Rose 1990; Sharma and Franzmann 2001). It could be then possible that taller neighbours would have made Douglas fir trees more difficult to locate by the Douglas fir needle midge, thus leading to a lower level of attacks. Those findings would be consistent with other studies having reported lower herbivore damage on

trees concealed by non-host neighbours (Floater and Zalucki 2000; Hughes 2012). The Apparency of Douglas fir trees was calculated, based on the height of their neighbours, regardless of their species. It should be noted, however, that the reduction in this Apparency was more often achieved in the presence of larch trees next to Douglas-firs, as *Larix x eurolepis* grows in height faster than Douglas fir at the juvenile stage (Supplementary material). This suggests that mixed stands of Douglas fir and larch might be less attacked by the midge, although not explicitly tested in this study and will be consistent with the associational resistance hypothesis. Conversely, Douglas fir was consistently more apparent in mixtures with slow-growing oaks and could have resulted in more gall midge damage, i.e. associative susceptibility. This leads us to suggest that it might not be species richness per se that confers greater resistance to mixed-species forests, but the identity and functional traits of the associated species included in the mixture (Jactel et al. 2021).

In addition to the effect of tree Apparency, we also found a positive effect of absolute tree height, with taller Douglas firs suffering from more needle damage than smaller ones. This result confirms the outcome of a study conducted by Castagneyrol et al. (2013) in a similar tree diversity experiment in France, showing that both tree Apparency and absolute tree height were positively correlated with damage by herbivorous insects on oaks. A simple explanation of this finding could be that taller trees are more likely to intercept insects moving in the environment than smaller trees. This effect will add to the increase Apparency of taller Douglas firs giving more visual cues than smaller ones. Another, although more speculative explanation of this pattern is related to the growth-differentiation balance hypothesis (Herms and Mattson 1992; Glynn et al. 2007), which suggests a physiological trade-off between growth and production of secondary metabolites in trees. Secondary metabolites in plants are often involved in defence mechanisms and, as such, fast growing trees are expected to produce fewer secondary metabolites, henceforth having lower defence and exhibiting higher level of herbivory (Lerdau et al. 1994; Massad 2013). Since all trees on the FORBIO tree diversity have the same age, it could be then possible that taller, fast-growing Douglas firs, had a lower amount of chemical defences and, thus, represented more suitable hosts for *C. pseudotsugae*. On the other hand, the Douglas fir midge belongs to the guild of gall makers that are known to manipulate the flow of photosynthetic products in the tree for their own benefit. Thus, the largest and, therefore, most vigorous Douglas-fir trees may be the best hosts for *C. pseudotsugae* as a primary pest.

Unexpectedly, we did not find any effect of Douglas fir density on mean damage by *C. pseudotsugae*. Plots with more abundant resource for the invasive pest were not more infested than plots with a lower concentration of host trees, contradicting the resource concentration hypothesis. This might be due to the small size of the plots in the FORBIO experiment (42 m × 42 m) (Bommarco and Banks 2003), the low level of active dispersion behaviour of the Douglas fir needle midge, or the overriding effect of Douglas fir Apparency that depends more on the species composition of the plots than on Douglas-fir density.

By contrast, the probability of a Douglas fir to be infected by *N. gaeumannii* was positively correlated with the density of Douglas fir in its direct neighbourhood. This result is consistent with recent studies that linked host tree proportion and pathogen transmission amongst neighbouring individuals (Pautasso et al. 2005; Field et

al. 2020). Increased resource availability has been theorised to be a key factor in the augmentation of infection by fungal pathogens amongst co-occurring, neighbouring, host trees (García-Guzmán and Dirzo 2004; Keesing et al. 2006, but see Hantsch et al. (2014)). For example, Hantsch et al. (2013) found in a tree diversity experiment in Germany that pathogen loads of several biotrophic fungi of European beech were higher when host trees were surrounded by conspecific neighbours. Ascospores of the Swiss needle cast are wind and water-splash dispersed (Ritóková et al. 2016). As such, it is likely that the transmission of the pathogen was facilitated by the shorter distances between infected and recipient neighbouring Douglas firs in pure plots.

Finally, we found that damage by *C. pseudotsugae* increased the probability of an infection by *N. gaeumannii*. Interactions between two or more invasive species in an area can result in invasion meltdown (Simberloff and Von Holle 1999; O'Loughlin and Green 2017), where a non-native species may facilitate the arrival or establishment of a subsequent invader. It is possible that the presence of *C. pseudotsugae* had indirect effects on the probability of infection by the fungal pathogen, by making Douglas fir more susceptible to colonisation. For example, Meyer et al. (2015) showed that abandoned galls of the chestnut gall wasp *Dryocosmus kuriphilus* could be colonised by the virulent form of the chestnut blight fungus *Cryphonectria parasitica*, which could lead to increased load of virulent inoculum in forests. Gossner et al. (2021) also showed that feeding damage by the weevil *Orchestes fagi* led to higher infection rates of beech leaves by the pathogenic fungus *Petrakia liobae*, by providing entry ports for fungal colonisation. In line with these studies, Douglas firs with a higher amount of galls could be more prone to a subsequent infection by the Swiss needle cast fungus *N. gaeumannii*. However, the opposite may also occur, with the Douglas fir needle midge causing more damage on Douglas firs infected by *N. gaeumannii*. Indeed, numerous studies have already shown that infections by pathogens can lead, directly or indirectly, to a greater susceptibility of trees to insect damage (Stout et al. 2006; Fernandez-Conradi, Jactel et al. 2018b; Moreira et al. 2018). Furthermore, we cannot rule out the possibility that both damage by the Douglas fir needle midge and infection by the pathogen responded to common tree traits that were not measured in this study, but could explain the co-linearity between the two variables. Finally, since the experimental design of this study did not allow us to determine precisely which of the pest or the pathogen initially affect focal Douglas fir trees, it would be interesting to better investigate the temporal dynamic behind the invasion by those two pests and pathogens and the effect that tree diversity could have on the mitigation of invasion meltdown.

Conclusions

Our study provides indirect support of the associational resistance hypothesis of mixed forests against exotic pests and pathogens. In the case of Douglas-fir Swiss needle cast, the effect was mediated by host dilution amongst heterospecific, non-host tree neighbours. In the case of Douglas-fir midge, the resistance effect was due to a reduction in host tree Apparency by taller neighbours. It should be noted, however, that the reduction of infestations by these exotic organisms was not general in mixed stands and might

mainly depend on the species composition of the mixtures. In particular, the need to incorporate tree species growing faster than Douglas-fir, such as hybrid larch, could be investigated as a mean of reducing pest damage. We also demonstrated for the first time a positive interaction between Swiss needle cast and Douglas fir needle midge. This underlines the necessity to develop a holistic approach to the problem of biological invasions in forests and, in particular, to look for generic prevention methods, amongst which the increase of forest diversity appears to be promising.

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